
Antifouling Activity of Prodigiosin from Estuarine Isolate of *Serratia marcescens* CMST 07

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Abstract

Microbial biofilms on the surfaces of man-made structures in the marine environment cause serious problems for marine industries. Currently used heavy metal-based toxic antifoulants has created environmental problem, which mandates the necessity of “eco-friendly” antifoulants. Marine-based microbial secondary metabolites are promising potential sources of nontoxic antifouling compounds. In the present study, we have investigated the antifouling potentials of bacterial red pigment prodigiosin extracted from *Serratia marcescens* CMST 07. Prodigiosin was showed high antifouling activity against marine fouling bacteria like *Alteromonas* sp. and *Gallionella* sp. minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the pigment was about 6.75 and 12.5 µg/ml respectively against *Alteromonas* sp. LD₅₀ of prodigiosin against artemia (artemia toxicity study) was about 50 µg/ml. Prodigiosin significantly ($P < 0.01$) inhibits cyanobacterial adhesion on glass surface, which augments the possibility of using bacterial pigments as the source of antifouling compounds for controlling the fouling problem in the marine environments.

Keywords

Antifouling • Bactericidal • Prodigiosin • *Serratia marcescens*

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Introduction

Marine biofouling is a serious problem caused by the accumulation and settlement of microbial slimes, diatoms, barnacles, tunicates, bryozoans etc., on the hulls of seafaring vessels (Clare 1996; Abarzua et al. 1999; Bhosale et al. 2002; Railkin 2003; Rasmussen and Ostgaard 2003). Biofouling on ship results in an increase in roughness of the hull that increases frictional resistance leads

to increased fuel consumption and associated environmental compliances (Depree 2006). Globally, billions of dollars are spent annually to control fouling on a variety of objects that are placed in marine environment. Currently available marine antifoulants are heavy metal-based that possess hazardous environmental problems (Bellas 2006; IMO 2007; Qian 2010; Thomas and Brooks 2010). Extension of this research area is essential to identify novel effective non-toxic compound having potent anti-micro and macro fouling properties. These biogenic compounds could also be used effectively for future development of antifouling paints (Hellio et al. 2001; Fusetani 2004; Greer et al., 2003). Naturally, marine environments harbor highly diverse microbial communities, which possess functionally undesirable and unexplored potentials (Whitman et al. 1998; Rappe and Giovannoni 2003), and they produce a variety of chemical deterrents for their defense purposes (Ren et al. 2001; Kubanek et al. 2002; Paul and Puglisi 2004). These bioactive compounds of marine microbial origin exhibit antifouling activity against variety of micro and macro foulants (Hellio et al. 2001).

Prodigiosin is a red-pigment produced as a secondary metabolite by *Serratia*, *Streptomyces*, *Pseudomonas*, *Pseudoalteromonas*, and few other bacteria, which share a common pyrrole dipyrromethene core structure and have a wide variety of biological properties, including antibacterial, antifungal, immunosuppressive, and anticancer activities (Bennett and Bentley 2000; Montaner and Perez-Thomas 2003). The present investigation reports the potential of red pigment prodigiosin extracted from estuarine bacteria *Serratia marcescens* CMST 07 for controlling the growth of marine micro and macro foulants.

Materials and Methods

Bacterial Strains

Red pigment prodigiosin producing *Serratia marcescens* CMST 07 (estuarine isolate) was

obtained from Centre for Marine Science and Technology, Manonmaniam Sundaranar University, Rajakkamangalam, and the marine fouling bacteria such as *Bacillus* sp., *Pseudomonas* sp., *Alteromonas* sp. and *Gallionella* sp. were obtained from Department of Zoology, Scott Christian College, Nagercoil. All bacterial cultures were stored in ZoBell marine agar (Himedia, India) at 4 °C.

Production, Extraction, and Characterization of Prodigiosin

Serratia marcescens was grown in nutrient broth (Giri et al. 2004) with 1.5 % NaCl at room temperature in shaking condition (150 rpm) for 24 h and further incubated at 28 °C for 72 h in static condition under dark. After the incubation, cells were harvested by centrifugation at 8,000 rpm for 15 min (Remi, India). Prodigiosin was extracted using chloroform: Methanol mixtures of increasing polarity (2:1 and 1:2 v/v), until the solution remains colorless (Nakashima et al. 2005). The crude extract was evaporated to dryness and the amount of pigment obtained on a dry weight basis was calculated. The resulting product was identified as prodigiosin by UV-visible spectrophotometry in the range 200–700 nm in 95 % ethanol and further subjected to thin layer chromatography (TLC) for further purification using the mixture of chloroform and methanol (9:1) as the solvent system (Casullo de Araujo et al. 2010). RF value of the extract was compared with standard prodigiosin (Sigma).

Antibacterial Assay

Antibiotic assays against four fouling bacteria were carried out using standard disk diffusion method (Bauer et al. 1966). The extracted prodigiosin pigment was filter sterilized by passing through Syringe driven filter (0.25 µm pore size; Himedia, India). The four different fouling bacteria were subcultured with marine broth (Himedia, India) for 12 h at 30 °C. Each bacterial strain was inoculated onto marine agar plates and then

dried Himedia sterile disks (6 mm) impregnated with 20 µg of crude prodigiosin were positioned on them. Disk impregnated with DMSO was maintained as controls to determine possible inhibitory activity of the solvent. The diameter of the inhibition zone around each disk was measured after the incubation at 30 °C for 24 h.

Determination of Minimum Inhibitory Concentration and Minimum Bactericidal Concentration

The pigmented prodigiosin obtained from the *S. marcescens* was diluted with DMSO at the standard concentration (100 µg/ml) and then two fold serially diluted up to the final concentration of 3.275 µg/ml in marine broth of 32 well microtiter plates. Thereafter, 100 µl of inoculum at a concentration of 1×10^7 CFU/ml was added to each well (Basri and Fan 2005). The microtiter plates were incubated at room temperature for 24 h. The MIC values were taken as the lowest concentration of the extracts in the well of the microtiter plate that showed no turbidity after incubation. The turbidity of the wells in the microtiter plate was interpreted as visible growth of microorganisms. Following MIC determination, MBC was determined by subculturing an aliquot of 50 µl from each well showing no apparent growth. Least concentration of extract showing no visible growth on subculturing was taken as MBC.

Antifouling Assay

Biofilm Inhibition Assay

Different concentration of prodigiosin was used to evaluate the inhibition of biofilm formation. The overnight grown culture of biofilm bacteria was transferred to the microtitre plate (2×10^8 CFU/ml), and different concentrations of prodigiosin were added to each well to the final volume of 200 µl. The plates were incubated for 24 h at 37 °C, and then bacterial biofilm was evaluated using crystal violet staining method

(Maldonado 2007). Bacteria without prodigiosin treatment were used as control.

Artemia Bioassay

Brine shrimp *Artemia parthenogenetica* (KKT1) was collected from Centre for Marine Science and Technology, Manonmaniam Sundaranar University, Rajakkamangalam. Brine shrimps were cultured in seawater at 25 ± 2 °C for 2 days before bioassay. Different concentration of prodigiosin coated Petri dishes were filled with 15 ml filtered seawater. Approximately 15 larvae were transferred to each plate and prodigiosin free plates were used as control. The plates were incubated at 25 ± 2 °C for 72 h with a light–dark cycle of 13:11 h. Dead and immobile larvae were counted after 24 and 48 h, respectively.

Cyanobacteria Adhesion Assay

Pure culture of the marine cyanobacteria *Synechococcus* sp. was cultured in sterile ASN III medium. The influence of prodigiosin on the growth of *Synechococcus* sp. was investigated by the direct contact test. Sterile microscopic slide was coated with prodigiosin as the contact surface. Uncoated slides were used as control. Slides were immersed in sterile 100 ml seawater adjusted with nutrients (seawater enrichment media) and 8×10^7 cells/ml *Synechococcus* sp. was added and incubated for 48 h under a light source of 1,000 X with a light–dark cycle of 13:11 h at 28 °C. Attached cells were counted by microscopically at 450 X magnification.

Barnacle Settling Assay

Balanus amphitrite larvae were obtained from adult barnacles collected from the Colachal coast (April, 2010). They were continuously fed with the brine shrimp *Artemia salina*. Newly settled barnacles were kept in aquaria with flowing, filtered seawater. Larvae were collected

by filtration (450–490 mm mesh size filter). Larvae were transferred to a 1.5 L flask with aerated seawater (27 °C) and fed with microalgae (Clare 1996). The cyprid stage barnacles were used for settling study. Settling test was performed using sterile Petri dishes and different concentrations of prodigiosin were coated (from 50 to 200 µg/ml). After drying, the experimental Petri dishes were filled with (5 ml) filtered seawater for 3 days and the dishes were replenished with 5 ml of fresh filtered seawater and then 20 cyprids transferred to each and assayed for settlement after 3 days.

Statistical Analysis

All the experiments were performed in triplicates to ensure probability and reproducibility of the results. One-way ANOVA analysis was used to test for significant differences between the concentration of prodigiosin on antifouling activity against fouling bacteria, artemia survival, attachment of cyanobacteria, and barnacle bioassay.

Results

The crude red pigment extracted from *S. marcescens* was purified by TLC and had the same *R_f* value (0.89) as compared with prodigiosin reference material (Fig. 1). The maximum absorption of the pigment was analyzed using UV–visible spectrophotometer at 531 nm.

Antibacterial Activity of Prodigiosin

The extracted prodigiosin was assessed for the antibacterial activity against different fouling bacteria (Figs. 2, 3). Prodigiosin exhibited a broad range of antibacterial activity as it inhibited both Gram-positive bacteria (*Bacillus* sp. 8.3 ± 1.52) as well as Gram-negative bacteria (*Alteromonas* sp. 16.3 ± 2.08 ; *Gallionella* sp. 9.3 ± 1.15 ; *Pseudomonas* sp. 6.3 ± 1.52). Further, the MIC of red pigment from *S. marcescens*

against biofouling bacteria was examined to determine the lowest concentration of antibacterial material require to inhibit cell growth completely (Table 1). Red pigment at a concentration of $12.5 < \text{mg/l}$ had showed antibacterial activity against *Alteromonas* sp., while *Gallionella* sp. was completely inhibited at a concentration of $25 < \text{mg/L}$. *Bacillus* sp. and *Pseudomonas* sp. were completely inhibited at higher concentration (100 mg/l). The MBC values of prodigiosin against different fouling bacteria were examined and shown in Table 1. Prodigiosin exhibited the low value of MBC as no viable cell growth was observed for *Alteromonas* sp. and *Gallionella* sp. at concentrations of 25 µg/ml and 50 µg/ml respectively on the solid medium. For *Bacillus* sp. no viable cell growth was observed at a concentration of 100 µg/ml, whereas viable growth was observed for *Pseudomonas* sp. even at the higher concentration tested in this study.

Antifouling Against Fouling Bacteria

Antifouling activity of prodigiosin was examined for its ability to inhibit fouling potential of fouling bacteria and shown in Fig. 4. Prodigiosin inhibits the biofilm formation at the concentration of 100 µg/ml against *Alteromonas* sp. and 200 µg/ml against *Gallionella* sp. For *Bacillus* sp. more than 90 % of the biofilm formation was inhibited at a concentration of 200 µg/ml, while only 40 % inhibition was observed against *Pseudomonas* sp. even at the higher concentration.

Artemia Survival Study

Brine shrimp lethality test has been previously used to evaluate bioactivity of the new metabolite (Meyer et al. 1982). Similarly, the inhibitory effect of prodigiosin on the survival of brine shrimp *Artemia* was studied at different concentrations of extracted red pigment (Fig. 5). Compare to control low concentration of prodigiosin also induces mortality of *Artemia* larvae. Prodigiosin at 50 µg/ml concentrations have shown 50 % (46.67 %) lethal effects after

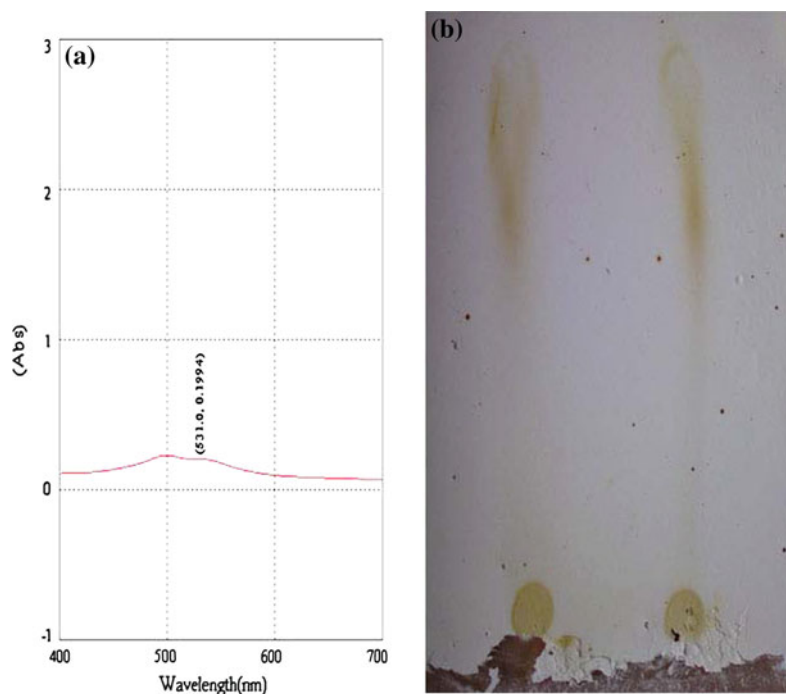


Fig. 1 Characterization of red pigment prodigiosin. **a** UV Spectrophotometric characterization of prodigiosin. **b** TLC analysis of prodigiosin. *M* standard prodigiosin (*Sigma*); *S* extract from *S. marcescens* cmst 07

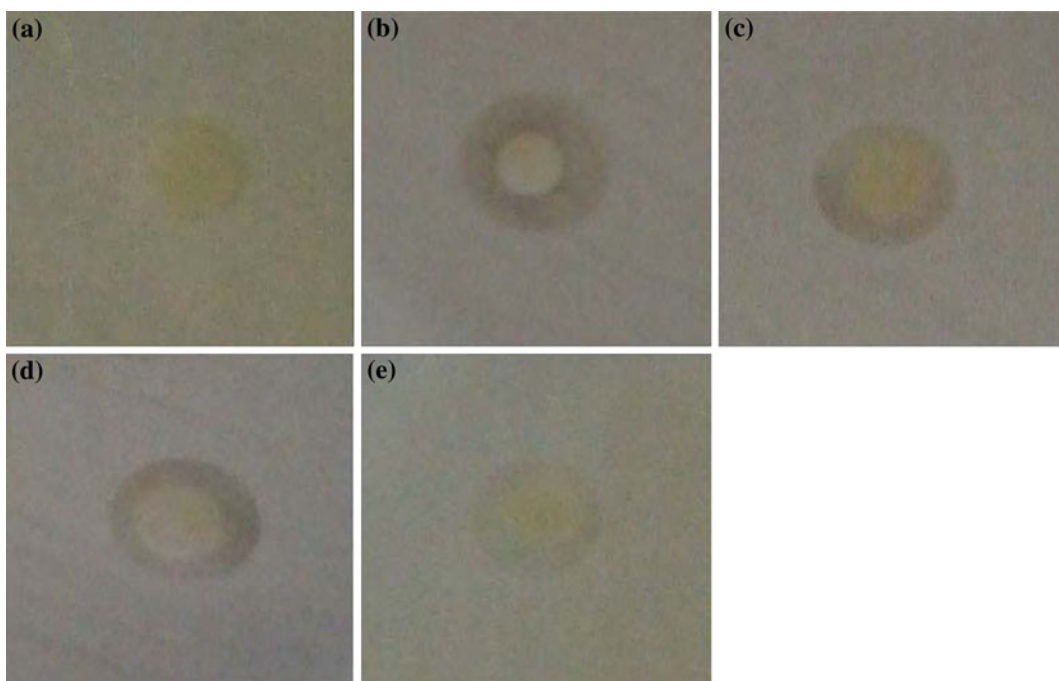


Fig. 2 Antibacterial activity of prodigiosin against marine fouling bacteria (10 µg/disk). **a** control, **b** *Alteromonas* sp., **c** *Gallionella* sp., **d** *Bacillus* sp. **e** *Pseudomonas* sp

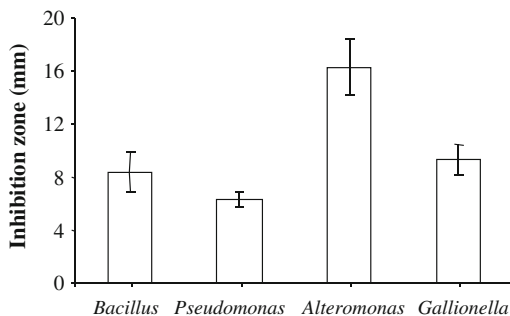


Fig. 3 Antibacterial activity of prodigiosin. Sterile antibiotic disk with 20 μg of prodigiosin was placed on agar with respective bacteria. All values are mean of three individual replicates with $\pm\text{SE}$

24 h of exposure, which can be considered as LD_{50} . Exposure to potassium dichromate was treated as positive control that exhibited significant lethality (LC_{50} value < 1.0 mg/ml) against the brine shrimp (data were not shown). With the comparison to the positive control, prodigiosin had shown higher lethality against *Artemia* survival.

Anti-algal Assays

The inhibitory effect of prodigiosin against fouling prokaryotic algae was assayed using the cyanobacteria *Synechococcus* sp. and the results have demonstrated that the concentration-dependent inhibition of cyanobacterial attachment on glass surfaces by prodigiosin (Table 2, Fig. 6). Red pigment obtained from estuarine bacterium.

S. marcescens had showed anti-cyanobacterial activity against *Synechococcus* sp. at 25–50 $\mu\text{g}/\text{ml}$ concentrations (ID_{50}).

Barnacle Settling Assay

Inhibition of larval settlement on solid surfaces by marine bacteria is commonly found in seawater (Wieczorek and Todd 1998). Indeed, settlement inhibition assays using barnacle cyprids have been used routinely to examine the antifouling properties of synthetic and natural

compounds. Effect of red pigment on barnacle setting was performed with *Balanus amphitrite* larvae and the results exhibited significant inhibition of the settlement of *B. amphitrite* cyprids (Fig. 7). When the cyprids were exposed to various concentrations of prodigiosin ranging from 50 to 200 $\mu\text{g}/\text{cm}^2$ larval settlement was inhibited in a dose-dependent manner. EC_{50} was between 100 and 200 $\mu\text{g}/\text{cm}^2$.

Discussion

The present investigation has aimed at finding alternative solution to the problem of marine biofouling, a serious problem faced by maritimes industries (Abarzua et al. 1999) and leads to enormous economic losses worldwide. Red pigment prodigiosin producing *S. marcescens* was used in this antifouling study, which was obtained from Centre for Marine Science and Technology, Manonmaniam Sundaranar University, Rajakkamangalam. The results obtained using prodigiosin of *S. marcescens* against biofouling validate the broad antibacterial potentials of the red pigment and are in agreement with the previous literature revealed the inhibitory effect of prodigiosin against both Gram-positive and Gram-negative bacteria (Mekhael and Yousif 2009; Samrot et al. 2011). Mekhael and Yousif (2009) have shown higher inhibitory effect of prodigiosins against Gram-positive bacteria than Gram-negative bacteria, whereas in the present study prodigiosin has higher activity against Gram-negative *Alteromonas* sp. and *Gallionella* sp. than Gram-positive *Bacillus* sp. Samrot et al. (2011) have reported that ethanol: HCl extract of *Serratia* has antibacterial activity and its zone of inhibition was higher against both Gram-negative (*E. coli* and *Pseudomonas* sp.) and Gram-positive (*S. aureus*) bacteria.

The results of MIC and MBC assays were clearly demonstrated the potentiality of red pigment prodigiosin as an effective antibacterial compound against fouling marine bacteria. It is known that the antibacterial activity of prodigiosin is the result of their potential to pass through the outer membrane and to their

Table 1 Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of prodigiosin against biofilm bacteria

Organism	Prodigiosin concentration ($\mu\text{g/ml}$)					
	100	50	25	12.5	6.75	3.275
MIC						
<i>Bacillus</i> sp	—	+	+	+	+	+
<i>Pseudomonas</i> sp	—	+	+	+	+	+
<i>Alteromonas</i> sp	—	—	—	—	+	+
<i>Gallionella</i> sp	—	—	—	+	+	+
MBC						
<i>Bacillus</i> sp	—	+	+	+	+	+
<i>Pseudomonas</i> sp	+	+	+	+	+	+
<i>Alteromonas</i> sp	—	—	—	+	+	+
<i>Gallionella</i> sp	—	—	+	+	+	+

+ presence of bacterial growth, — absence of bacterial growth

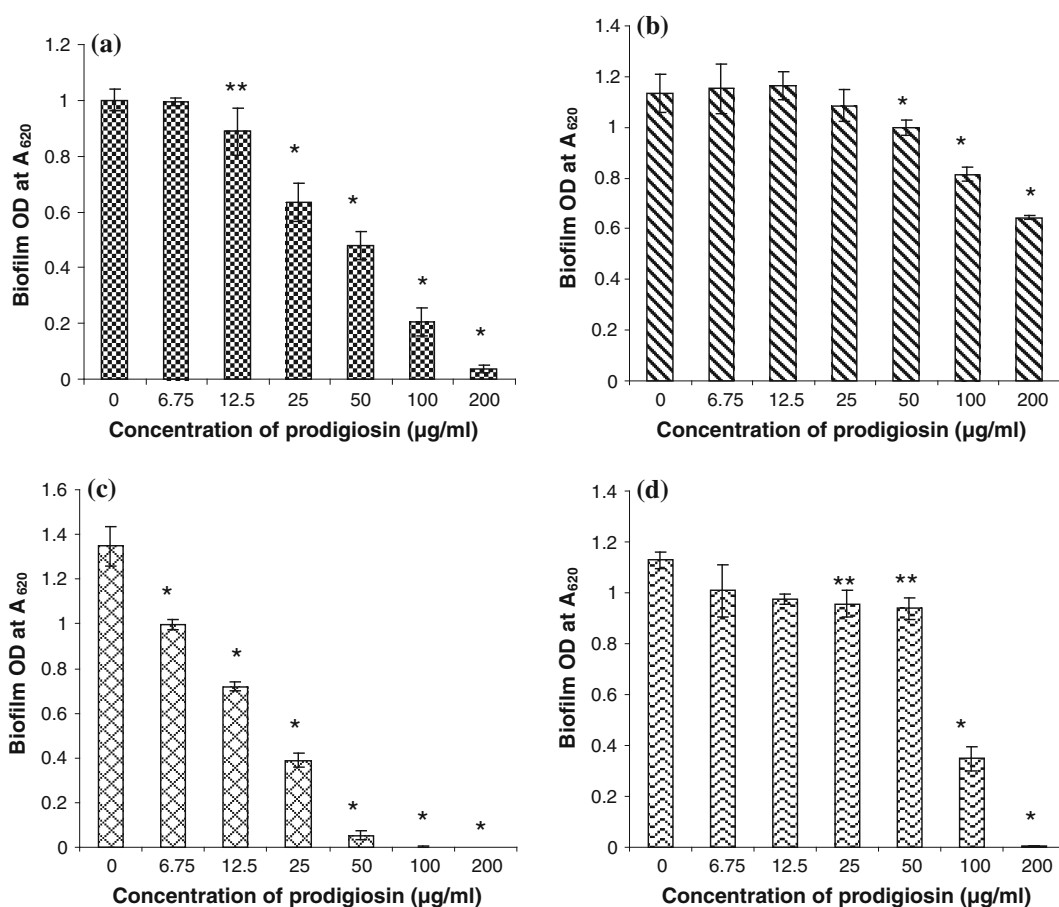


Fig. 4 Antifouling activity of prodigiosin against fouling bacteria. All values are mean values of three individual experiments with \pm SE. **a** *Bacillus* sp., **b** *Pseudomonas* sp., **c** *Alteromonas* sp., **d** *Gallionella* sp.

$P < 0.0001$ in ANOVA at different concentrations. * $P < 0.01$ significant, ** $P < 0.05$ significant in Tukey HSD test

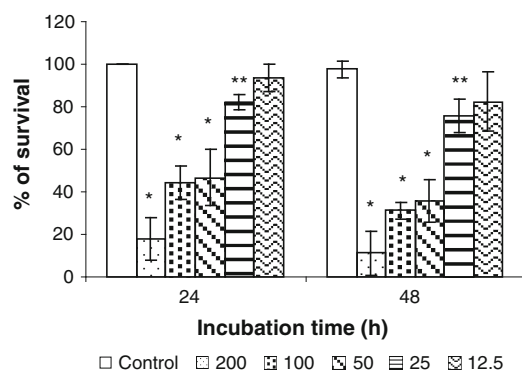


Fig. 5 Survival of artemia against concentration of prodigiosin. All values are mean of three individual replicates with \pm SD. One way ANOVA $P < 0.0001$ significant was observed. Asterisk $P < 0.01$, double asterisk $P < 0.05$ significant in Tukey HSD test

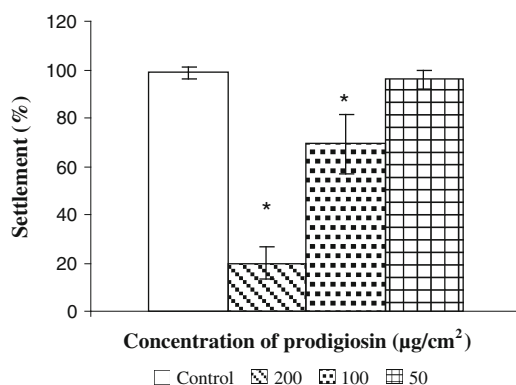


Fig. 7 Effect of prodigiosin against Barnacle settlement [after 3 days of incubation ($n = 25$)]. One way ANOVA $P = 0.913233$; Tukey HSD test Asterisk $P < 0.01$ significant

Table 2 Effect of prodigiosin on cyanobacterial adhesion on glass surface

Time (h)	Number of cyanobacterial (cells/ cm^2)					
	Control	200	100	50	25	12.5
24	66.25 \pm 14.40	7.75* \pm 2.21	11.5* \pm 2.64	56.75 \pm 17.87	59.75 \pm 19.37	62 \pm 14.30
48	189.75 \pm 38.24	16.5* \pm 4.12	61.25* \pm 9.74	106.25* \pm 10.04	142.25 \pm 34.86	191.5 \pm 34.81

All values are mean of triplicates with \pm standard deviation. One way ANOVA $P < 0.0001$; Tukey HSD test * $P < 0.01$ significant

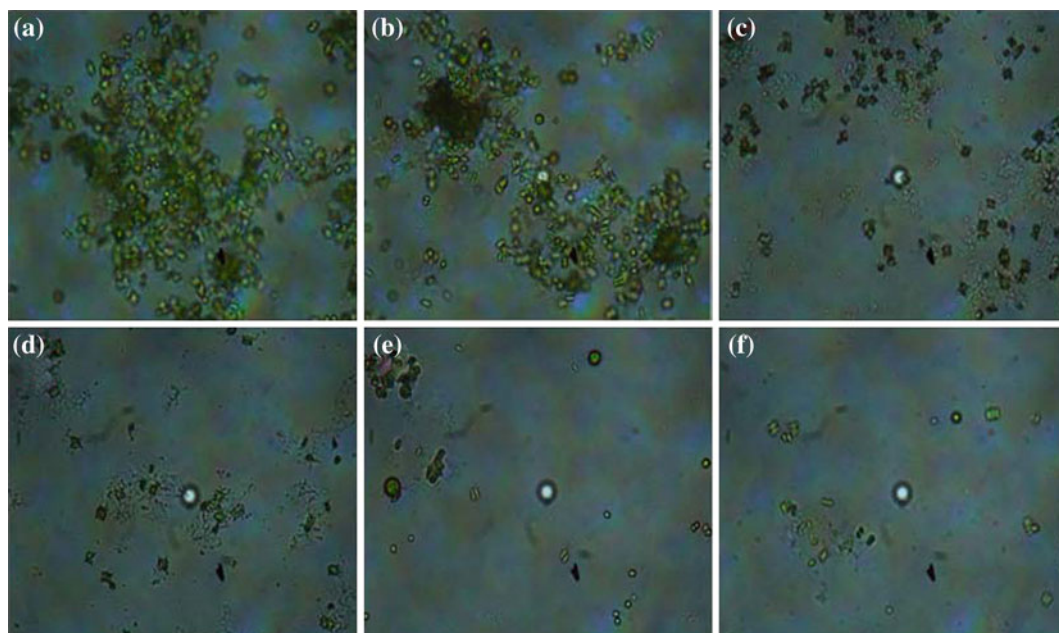


Fig. 6 Effect of prodigiosin against cyanobacterial adhesion (slide adhesion assay (400 X magnification). **a** control, **b** 12.5 $\mu\text{g}/\text{cm}^2$, **c** 25 $\mu\text{g}/\text{cm}^2$, **d** 50 $\mu\text{g}/\text{cm}^2$, **e** 100 $\mu\text{g}/\text{cm}^2$, **f** 200 $\mu\text{g}/\text{cm}^2$

capacity for inhibiting target DNA modulating enzymes, such as DNA gyrase and topoisomerase IV, which inhibit the cell growth (Berlanaga and Vinas 2000). Since, the antibacterial activity of a compound may depend on the destruction of the physical structure or the inhibition of any metabolic reaction in a microorganism, it seems that the presence and the level of the antibacterial activity of the red pigment varied significantly with the type of fouling bacteria used. Furthermore, the red pigment caused growth inhibition along with bactericidal activity, it suggests that the red pigment is an effective antibacterial agent.

Marine-based microorganisms have lot of unexplored potentials; however, the exploration of marine resources for antifouling compounds is very limited (Dobretsov et al. 2006; Paul et al. 2006). The present investigation reveals the biological activity of red pigment prodigiosin against marine fouling bacteria that were exposed into the same ecological conditions. Interestingly, prodigiosin extracted from the *S. marcescens* had showed broad spectrum of antifouling activity against biofoulants of marine environment like *Alteromonas* sp., *Gallionella* sp. and *Pseudomonas* sp. by means of significant decrease in the adherence and biofilm formation of fouling bacteria compared with control. Similarly, antibacterial activity of marine organisms against biofilm forming bacteria is reported in earlier (Wilsanand et al. 1999; Marechal et al. 2004). A new yellow pigment has been isolated from the marine bacterium *Pseudoalteromonas tunicata* and identified as a new member of the tambermine class of compounds has antifouling activity against marine fouling (Egan et al. 2002; Frank et al. 2005). Considering the results of this study may give immense values of marine-based natural products and the needs for the collection of novel marine resources for discovery and development of effective natural products.

Assaying anti-algal activities by prodigiosin against fouling cyanobacteria *Synechococcus* sp. have been demonstrated concentration-dependent inhibition of cyanobacterial attachment on glass surfaces. Similarly, Egan et al. (2002) have

been identified a compound from the marine bacterium *P. tunicata* exhibited anti-algal activity that inhibits settlement of spores of *Ulva lactuca*. Another marine bacterium *Alteromonas* sp. produced 2-n-Pentyl-4-quinolinol, which inhibited the growth of diatoms even at nanomolar concentrations (Long Richard et al. 2003). Kang et al. (2005) have reported anti-cyanobacterial effects *Pseudomonas putida* against *Microcystis aeruginosa*. Furthermore, effect of red pigment on barnacle setting was performed with *Balanus amphitrite* larvae and the results were exhibited significant inhibition of the settlement of *B. amphitrite* cyprids. Thus, results of these bioassay studies clearly indicate the positive response of prodigiosin as an antifouling natural metabolite, which are in agreement with previously reported studies of *B. amphitrite* settlements (Rittschof et al. 1984; Oclarit et al. 1994; Maki et al. 1998; Lau et al. 2003).

Based on this study, it seems that bacterial red pigment prodigiosin has the potential to inhibit the growth of marine fouling bacteria, cyanobacteria, and invertebrates. The findings of the study proved that, the red pigmented prodigiosin has an alternative to chemical antifoulants against marine micro and macro foulants, which encourage to developing a novel broad spectrum antifouling formulation in future. Furthermore, molecular studies require proving the mechanism of the compound act as an effective antifouling.

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